Bringing green valleys to aquaculture

Alfalfa white protein extract as a novel ingredient in feed for rainbow trout

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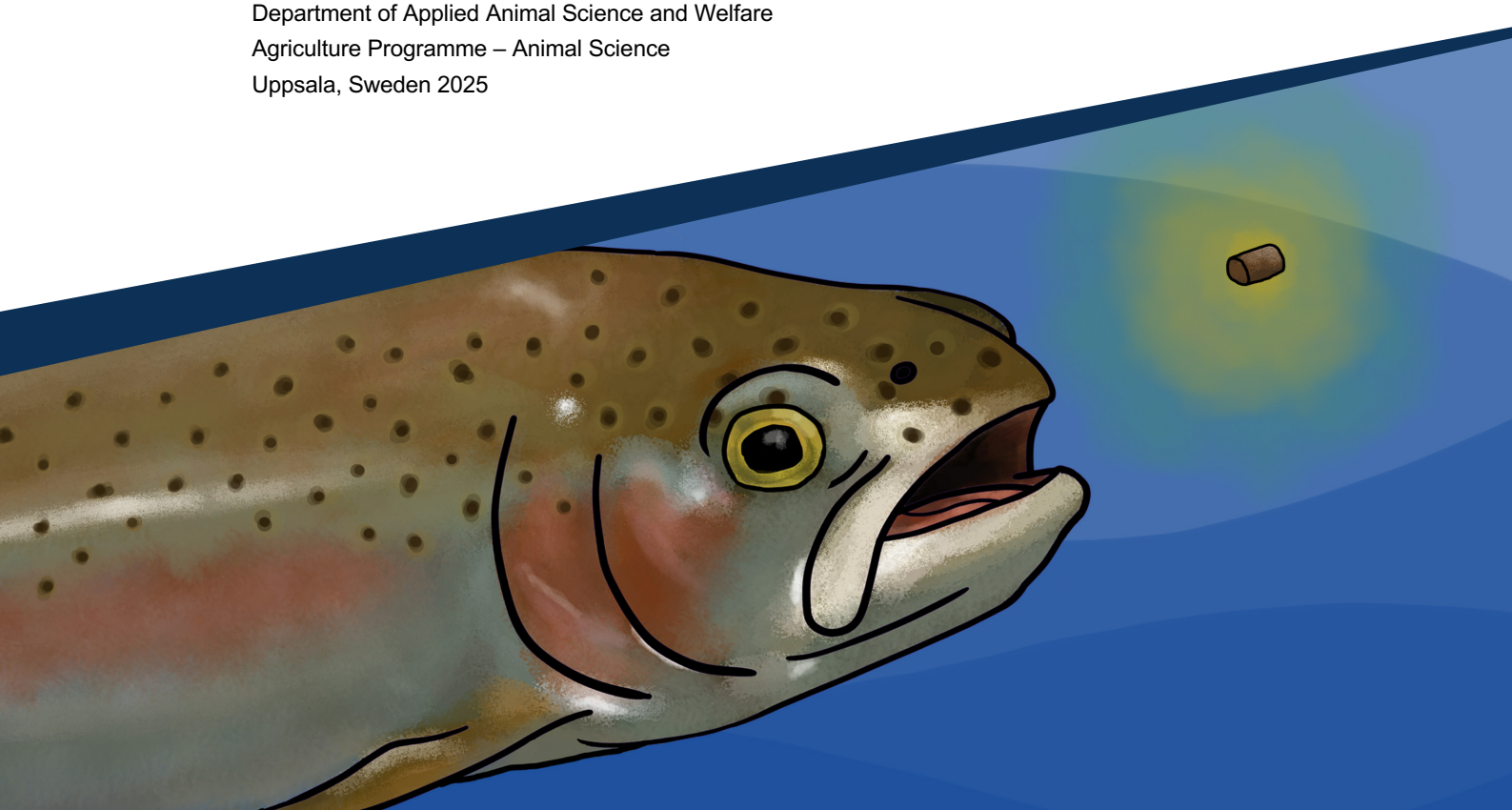

Bringing green valleys to aquaculture. Alfalfa white protein extract as a novel ingredient in fish feed for rainbow trout.

Gröna dalar till akvakulturen. Alfalfa vitt proteinkoncentrat som ny ingrediens i fiskfoder till regnbågslax.

Nathalie Navarrete

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Abstract

Aquaculture is quickly expanding to meet global protein demand and has untapped potential to provide food security for a growing human population. However, the most common protein sources used to produce feed for farmed fish, fish meal and soybean concentrate, are associated with grave environmental consequences. Thus, sustainable expansion of the fed aquaculture sector relies on discovering novel protein sources. This study explores if white protein (WP) concentrate extracted from alfalfa (*Medicago sativa*) through green biorefinery may be a promising alternative. A 47-day feed trial was conducted on rainbow trout (*Oncorhynchus mykiss*) to assess the effect on physical pellet quality, growth performance and fish health. Four experimental diets were produced: one fish meal-based control diet (CTRL) and three diets replacing fishmeal with WP at 5%, 10% and 20% of total feed composition content (WP5, WP10 and WP20). The WP20 diet was pre-extruded.

The physical pellet quality indicators tested were water stability, bulk density, expansion ratio and pellet hardness. The WP20 diet had significantly higher water stability than all other diets in nearly every period tested (30, 90 and 180 minutes), followed by CTRL, WP5 and WP10. As there is no clear correlation between increased WP inclusion and increased water stability, the pre-extrusion of the WP20 diet has likely improved its water stability properties. There were no significant differences between diets in either bulk density or expansion ratios. However, the expansion ratios gradually reduced with higher inclusion of WP, indicating pellets were more compact when containing WP. Pellet hardness was also reduced as WP levels increased, meaning that pellets containing WP were softer. The effect of pre-extrusion on physical pellet quality should be investigated further.

No significant differences were identified between fish fed with the CTRL, WP5 and WP10 diets in any of the fish growth performance indicators tested. However, gradual decreases in almost all parameters tested were observed with increasing inclusion of WP. The WP20 diet had significantly lower Fulton's condition factor, weight gain, feed intake and SGR, and the highest FCR. Furthermore, a gradual increase of WP in the feed formulations generated decreasing ADCs of dry matter and crude protein. Future studies should further investigate the impact of WP on fish health parameters. Yet, these results indicate that WP could be considered for inclusion in fish feed for rainbow trout at 5% and 10%. Longer study durations with rainbow trout at varying life stages should be performed to confirm these results.

Keywords: alfalfa white protein extract, rainbow trout, physical pellet quality, growth performance, hematocrit levels

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Abbreviations

ADC	Apparent digestibility coefficient
ANF	Antinutritional factor
CF	Crude fiber
CL	Crude lipid
CP	Crude protein
CTRL	Control diet
DD	Die diameter
DM	Dry matter
DO	Dissolved oxygen
FCR	Feed conversion ratio
FI	Feed intake
FL	Final length
FM	Fish meal
FW	Final weight
GE	Gross energy
HIS	Hepotosomatic index
K	Fulton's condition factor
PW	Pellet width
SBM	Soybean meal
SD	Standard deviation
SGR	Specific growth rate
SLU	Swedish University of Agricultural Sciences
SW	Start weight
VSI	Viscerosomatic index
WG	Weight gain
WP	White protein

1. Introduction

1.1 Aquaculture and food supply

In recent decades, there has been a significant search for alternative and novel protein sources in human food and animal feed. The human population is estimated to grow to more than 9.5 billion by the year 2050 and, consequently, food demands are increasing (UN 2013). By then, based on current trajectories, fish consumption is expected to continue to expand and might double (Naylor et al. 2021a; Falcon et al. 2022; FAO 2024). As fisheries catches have stagnated since the 1980s, aquaculture has increased to meet the growing demand for seafood. As of 2022, aquaculture production of aquatic animals has surpassed the capture fisheries production, and 51% of the 185 million tonnes of total aquatic animals produced came from aquaculture (FAO 2024). Finfish species comprise the largest part of aquatic animal production and are mostly farmed in inland environments (FAO 2024).

Aquaculture is a relatively young industry and has rare potential to work toward sustainable expansion (Gephart et al. 2021). Compared to other animal food production systems, particularly that of ruminants, fish aquaculture has been discussed as a biologically efficient way to produce animal protein (MacLeod et al. 2020). This is due to fish's high fertility and low feed conversion ratios (FCRs) compared to other animal food systems. The biological efficiency of aquaculture, in turn, generates relatively low levels of greenhouse gas emissions (MacLeod et al. 2020). If aquaculture is to become more sustainable, some challenges must be addressed (Gephart et al. 2020, 2021). Among these is the need to produce sustainably sourced fish feed (Willett et al. 2019).

1.2 Feeds in aquaculture

Fed aquaculture constitutes approximately two-thirds of all aquaculture and is continuously increasing (FAO 2024). High-quality feed is essential in fed aquaculture to promote growth, health and successful fish reproduction (Lall & Dumas 2022). Feeding behaviours and nutritional requirements differ broadly between species, but for most, especially carnivorous species, high levels of quality protein in feed is beneficial (NRC 2011; Lall & Dumas 2022).

Fishmeal (FM) has long been considered the ideal protein source for use in aquaculture feeds (Teles et al. 2020). FM contains high levels of protein and other nutrients, has a balanced essential amino acid profile and is considerably palatable for fish (FAO 2024; Glencross et al. 2024; Serra et al. 2024). Some FM is used to feed pigs, poultry, and other animals, but the vast majority goes towards aquaculture (FAO 2024). FM is usually produced from whole, partial or by-products from small pelagic fish species, and the nutritional content of the FM will change depending on the raw material input. The nutritional content may also vary in feed intended for carnivorous fish compared to lower-trophic species (Hua et al. 2019). The dependence on FM remains strong as the aquaculture industry continually grows. Still, despite this, there is an overall downward trend of its use in aquaculture feeds, brought on by associated negative impacts on the environment (Naylor et al. 2021a; b; FAO 2024). FM has also become more expensive, and thus, other protein sources are increasingly used in feeds (Jannathulla et al. 2019; Mugwanya et al. 2023). Feed is costly and can comprise up to 70% of the total production cost in aquaculture (FAO 2024), and protein ingredients are generally the most expensive (Teles et al. 2020).

Over the years, FM in fish diets has gradually been replaced by plant-derived protein from soybeans (*Glycine max*) (Naylor et al. 2021a; Serra et al. 2024). The beans are used primarily to extract high-quality oil, but the remaining by-product can be made into soybean meal (SBM) (Ma 2015; Falcon et al. 2022). However, the use of SBM in fish feeds is somewhat limited due to the presence of antinutritional factors (ANFs) (Krogdahl et al. 2022). The function of ANFs is to protect the plant against external attacks, e.g. from insects or fungi (Krogdahl et al. 2022). In salmonids, ANFs found in soy have been seen to reduce gut function and, subsequently, nutrient utilisation (Krogdahl et al. 2022). Further processing of SBM into concentrates or isolates can decrease the levels of ANFs in the soy ingredient (Alarape et al. 2024) and make it more suitable in fish feed. Yet, soy-derived ingredients are also controversial for other reasons, such as their association with environmental and socioeconomic consequences (da Silva et al. 2021; Song et al. 2021). Deforestation and change in land use to facilitate soybean production risks ecosystem damage on land and in aquatic environments, particularly in South

America (Song et al. 2021). Economic benefits at a local level due to the production have also been seen to have little positive spill-over effects on surrounding areas, while, unfortunately, deforestation does (da Silva et al. 2021). As soybean protein plays an increased role in aquaculture, demand and subsequent prices may increase (Naylor et al. 2021a; Falcon et al. 2022).

The aquaculture industry is working to reduce the dependence on FM and soybean protein. Therefore, there is research focused on identifying new sustainable protein sources (Lall & Dumas 2022; FAO 2024; Glencross et al. 2024). Among the considered ingredients are animal by-products, insect meals and algae (Aragão et al. 2022). One of the underutilised resources potentially suitable for generating protein for aquaculture involves protein extracts from perennial crops.

1.3 The alfalfa white protein extract

Alfalfa (*Medicago sativa*), colloquially known as lucerne, is a perennial plant which natively belongs to the Mediterranean region (Hadidi et al. 2023). Today, alfalfa grows all across Europe and is mainly used to feed livestock (Hadidi et al. 2023). The plant is high in protein and possesses good nutritional quality (Firdaous et al. 2017; Hadidi et al. 2023). Moreover, as alfalfa is widely available, it regrows annually and tolerates climate extremes and salinity while being easy to grow (Firdaous et al. 2017). Due to this, alfalfa is usually also inexpensive (Hadidi et al. 2023). Alfalfa has been recognised for its potential as a sustainable protein source for humans and other monogastric animals (Pirie 1972; Firdaous et al. 2017; Hadidi et al. 2023; dos Passos & Ambye-Jensen 2024). While not widely used in diets for fish, it has been researched to some extent in diets for Nile tilapia (*Oreochromis niloticus*) fingerlings, goldfish (*Carassius auratus*), yellow perch (*Perca flavescens*) and most recently, rainbow trout (Ali et al. 2003; Yanar et al. 2008; Coburn et al. 2021; Chen et al. 2024). However, the technology used to produce the alfalfa product tested in these studies varies greatly.

Processing the alfalfa crop is necessary to enhance its desirable properties and access the protein (Ali et al. 2003). One way to extract protein from alfalfa is through green biorefinery, which consists of several steps (Møller et al. 2021; Gaffey et al. 2023; dos Passos & Ambye-Jensen 2024). These steps vary, depending on the final goal product. To best recover the white protein fraction, freshly harvested alfalfa first enters a wet fractionation process (dos Passos & Ambye-Jensen 2024). Here, the plant cells are mechanically pressed and crushed through a screw press, separating the biomass into two fractions: the fibre press cake and the green juice (dos Passos & Ambye-Jensen 2024).

Fibre press cake is suitable as feed to ruminants, while green juice contains proteins and other soluble plant components of the biomass (Møller et al. 2021). The green juice can be used in biogas production (Møller et al. 2021), or a secondary fractionation processing step may be introduced to extract the proteins from the green juice (summarised in Figure 1). The proteins in the green juice can be categorised into two fractions, called *the green fraction* and *the white fraction* (dos Passos & Ambye-Jensen 2024). The green fraction is mostly made up of chloroplast proteins and lipids. The white fraction mainly consists of cytoplasm and is rich in the plant protein ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (dos Passos & Ambye-Jensen 2024).

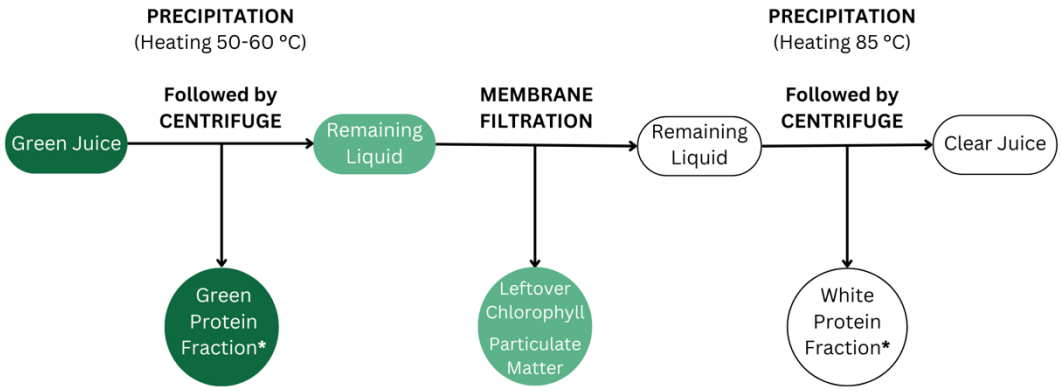

Figure 1. Overview of the biorefinery steps to extract the green and white protein fractions from green juice. (*) Both fractions are dried after centrifuging.

Heating the green juice at 50-60 °C precipitates the green fraction, which, following centrifuge, can be extracted from the liquid (dos Passos & Ambye-Jensen 2024). The remaining liquid will still contain the cytoplasmic proteins, but a microfiltration step is used to separate leftover chlorophyll and other particulate matter. Another round of heat treatment at 85 °C precipitates the white fraction, which can be extracted after centrifuging. Both the green and the white protein fractions are dried post-centrifuging (dos Passos & Ambye-Jensen 2024).

The white fraction is higher in protein than the green and more easily digestible for monogastric animals (Møller et al. 2021). In the previously mentioned feed trials, fish have shown acceptance of feed containing alfalfa, but growth parameters have declined at higher levels (Ali et al. 2003; Yanar et al. 2008; Coburn et al. 2021; Chen et al. 2024). The authors discuss this potentially being due to the presence of ANFs in the alfalfa plant, particularly saponins. However, as the extraction process of the white fraction is continuously refined (dos Passos & Ambye-Jensen 2024), it may become more suitable to include as a protein source in fish feed.

1.4 Assessment of novel ingredients in fish diets

How a new ingredient is assessed may affect how the quality of the ingredient is interpreted. Attempts have been made to standardise the process in keeping with developments within scientific ingredient evaluation methods and user expectations (Glencross et al. 2007; Glencross 2020). The same ingredient may perform differently depending on the exact origin, supplier or processing methods used to make the ingredient (Sørensen 2012). This includes a comprehensive consideration of several factors, such as an ingredient's characterisation, palatability and digestibility (Glencross 2020). Some feed ingredients may affect fish health, thus reducing nutrient retention and fish growth (Krogdahl et al. 2022).

Any new ingredient must be functional when used for feed production, transport, storage, and to meet species-specific needs (Glencross 2020). Few feed studies include physical pellet quality analysis despite its impact on the nutritional value of the feed (Aas et al. 2011; Sørensen 2012). Feed is typically made by extrusion, and the different physical properties of the pellets are carefully considered to ensure a final feed product which is durable and reaches the necessary standard (Sørensen 2012; Davis & Hardy 2022). There are a variety of tests that can be used to assess the quality, durability and behaviour of feed. Bulk density is a measure of pellets' capacity for oil absorption and buoyancy and is directly dependent on the expansion ratio of pellets during extrusion. Pellet hardness relates to the binder content in the feed formulation. Furthermore, water-stable pellets stay intact for longer in water and have less nutrient leaching, making it an important characteristic to consider (Sørensen 2012; White 2013; Davis & Hardy 2022).

1.5 Aim and hypothesis

This thesis explores the potential of including white protein extract from alfalfa as a new ingredient in fish feed for rainbow trout (*Oncorhynchus mykiss*). The study focuses on assessing the nutritional value of alfalfa white protein concentrate, its effects on physical pellet quality, and the potential in feeding trials with rainbow trout. A higher inclusion rate in fish feed will likely correlate with changes in both pellet quality and fish performance. Secondly, the study assesses the effects of increasing inclusion levels of protein concentrate on growth parameters and digestibility in a 47-day trial. The hypothesis is that the diets containing more alfalfa white protein will perform as effectively as the control diet. Thirdly, the haematological status of fish fed with experimental diets will be analysed to assess the effects on fish health and welfare. As the alfalfa white protein likely contains antinutritional components, it is hypothesised that there will be changes in haematological parameters.

2. Materials and methods

2.1 Experimental diets

Four experimental diets were formulated and used in the feed trial: one reference control diet (CTRL) based on FM and comparable to commercial feed and three diets where WP extract replaced FM at 5%, 10% or 20% of total feed content. Formulation of dietary recipes has been performed to overshoot the minimal nutritional requirement levels for rainbow trout, as established by NRC (2011). The diets are isonitrogenous, containing the same levels of protein, to make them comparable. The WP extract was provided by the Department of Biological and Chemical Engineering at Aarhus University, Denmark. In experimental feeds, vitamin and mineral premix, DL-methionine and Monocalcium phosphate were added to balance all diets. Yttrium oxide (Y_2O_3) was added as an inert marker for later use in calculating the apparent digestibility coefficient (ADC). See Table 1 for complete feed formulations.

Table 1. Formulations of experimental diets, expressed in % of the diet, on an 'as is' basis.

Diet	CTRL	WP5	WP10	WP20
Ingredient	**%**	**%**	**%**	**%**
Fish meal	35	30	25	15
Soy protein concentrate	21	21	21	21
Wheat gluten	10	10	10	10
Wheat meal	12.6	12.4	12	11
Fish oil	10	10	10	10
Rapeseed oil	8	8	8	8
White protein	0	5	10	20
Choline chloride	0.15	0.15	0.15	0.15
Yttrium oxide (Y_2O_3)	0.01	0.01	0.01	0.01
Vitamin mineral premix	1	1	1	1
DL-methionine	0.25	0.29	0.33	0.41
Monocalcium phosphate	2	2.2	2.5	3.4
Total	100	100	100	100

The feed was produced in the Feed Technology Laboratory at the Swedish University of Agricultural Sciences (SLU; Uppsala, Sweden) using a twin-screw Brabander Ketse 20/40 extruder (Brabender GmbH & Co. KG, Duisburg, Germany) in April and May 2024. An extruder barrel water injection was performed using a peristaltic liquid dosing pump, AgnThos 120U (AgnTjo's AB, Lidingö, Sweden). The WP20 diet was pre-extruded. A summary of the feed extrusion parameters used during feed production of experimental diets can be found in Table 2.

Table 2. Feed extrusion parameters during the production of the experimental diets, including temperatures in the five-barrel sections 1-5 in °C, pressure at the die (bar) and die diameter (mm).

Diet	CTRL	WP5	WP10	WP20
Barrel section 1 (°C)	100	100	100	100
Barrel section 2 (°C)	120	120	120	120
Barrel section 3 (°C)	130	130	130	130
Barrel section 4 (°C)	120	120	120	120
Barrel section 5 (°C)	100	100	100	110
Pressure at the die (bar)	24.6	24.7	31.2	38.3
Die diameter (mm)	2	2	2	2

After extrusion, the pellets were dried overnight in a drying oven (Elvärmedetaljer, Skurup, Sweden) at room temperature and then, using a GVC-10-mini vacuum coating system (Amandus Kahl GmbH & Co. KG, Reinbek, Germany), coated with lipids. Additionally, the WP20 feed was double-coated, as it did not successfully absorb all of the oil upon first coating. See Appendix 1, Figure 10 for images of the final feed products.

2.2 Feed chemical analysis

The proximate chemical composition of feed samples was analyzed at the Department of Applied Animal Science and Welfare at SLU, Uppsala, Sweden, in April and May of 2024. Chemical analyses were performed on all four feeds, measuring dry matter (DM), ash, crude protein (CP), crude lipid (CL), crude fibre (CF), gross energy (GE), amino acids (AA) and minerals (see Table 3). DM was analyzed by heating feed samples at 103 ºC for 16 hours, then cooling in a desiccator before weighing. To establish the ash levels, the DM sample was heated in an oven at a temperature of 550 ºC for 3 hours. Afterwards, the sample was cooled in a desiccator and weighed. Total nitrogen (N) was ascertained using the Kjeldahl method, with the help of a 2520 Digestor, Kjeltec 8400 Analyser unit and an 8460-sampler unit (all from FOSS Analytical A/S, Hilleröd, Denmark). N was

then used to calculate CP using the formula N × 6.25 (NMKL, 1976). A Hydrotec 8000 and a Soxtec 8000 Extraction Unit (FOSS Analytical A/S Hilleröd, Danmark) were utilized to analyze CL, in accordance with the Official Journal of the European Communities (2009). The CF was determined according to protocol by Jennische and Larsson. GE was measured via an isoperibol bomb calorimeter (Parr 6300, Parr Instruments Co. Moline, IL, United States). Lastly, AA profiling was performed using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS) at Eurofins Biopharma Product Testing Sweden AB in Uppsala, Sweden.

Table 3. Feed chemical composition (g kg^{-1} DM unless otherwise stated) of experimental feeds.

Diet	**CTRL**	**WP 5**	**WP 10**	**WP 20[1]**
DM (%)	94.88	94.44	94.42	96.07
Ash	88.98	89.16	87.21	75.69
Crude protein	484.83	492.52	508.06	504.31
Crude fat	222.04	225.27	222.29	187.90
Crude fibre	14.13	9.22	8.27	9.64
GE (MJ kg^{-1} DM)	21.44	20.99	21.54	22.54
Sum of AA	406.96	412.5	409.95	438.01
Essential amino acids				
Arginine	25.50	26.40	26.60	23.60
Histidine	9.00	9.76	9.51	11.40
Isoleucine	17.00	17.80	17.30	19.70
Leucine	32.50	33.60	33.40	37.90
Lysine	27.80	27.70	27.40	27.80
Methionine	11.10	11.30	11.20	12.70
Phenylalanine	19.90	21.20	21.30	24.70
Threonine	16.60	17.70	17.40	19.80
Valine	19.50	20.20	20.20	23.40
Non-essential amino acids				
Alanine	22.40	22.50	22.30	23.60
Aspartic acid	38.40	39.70	39.50	43.10
Cysteine +Cystine	5.67	6.04	5.54	5.81
Glutamic acid	87.10	386.50	86.00	86.60
Glycine	23.90	23.60	23.40	23.50
Hydroxyproline	2.69	ND	ND	ND
Proline	27.20	27.70	28.20	28.90
Serine	20.70	20.80	20.70	20.50

ND = not detected

Yttrium was analysed at the Faculty of Biosciences at the Norwegian University of Life Sciences (NMBU, Ås, Norway) in September 2024. Samples were completely digested in a Start D Microwave Digestion System (Milestone Srl, Sorisole, Italy) using concentrated nitric acid (HNO_3) and hydrogen peroxide (H_2O_2). The pre-digested samples were analysed spectrophotometrically using a Microwave Plasma Atomic Emission Spectroscopy (MP-AES) 4200 (Agilent Technologies, Santa Clara, CA, USA).

2.3 Experimental fish and design

In this study, rainbow trout were obtained from Vattudalens Fisk AB and relocated to the Aquatic Facility of the Centre for Veterinary Medicine and Animal Science at SLU (Uppsala, Sweden). Fish were kept in a holding tank (2500 L) for ten days before the trial. After this, 480 fish weighing 20 ± 6 g were selected for the feed trial and evenly distributed with 30 fish per tank into 16 oval flow-through tanks with a volume of 200 L each. The fish were not fed on the first day post-weighing in order to allow them to acclimate to the new environment. The feeding trial began on the 6th of July 2024 and was finalized on August 22nd, 2024, lasting for a total of 47 days.

During initial weighing and distribution, fish were anaesthetized in a 10 L bucket using tricaine methanesulfonate Syncain MS-222 (Syndel, Ferndale, Washington, USA) at a concentration of 40 mg L^{-1}, buffered with sodium bicarbonate. After they were immobilized, as measured by the lack of reaction to external stimuli, the fish were weighed using a scale (Mettler PM4600 DeltaRange Balance). If within the predefined weight range, the fish were then measured with a ruler (Figure 2). Fish chosen for the trial were moved to recover in their assigned trial tank. Each tank had been randomly selected for one of the experimental diets, with four tanks per diet.

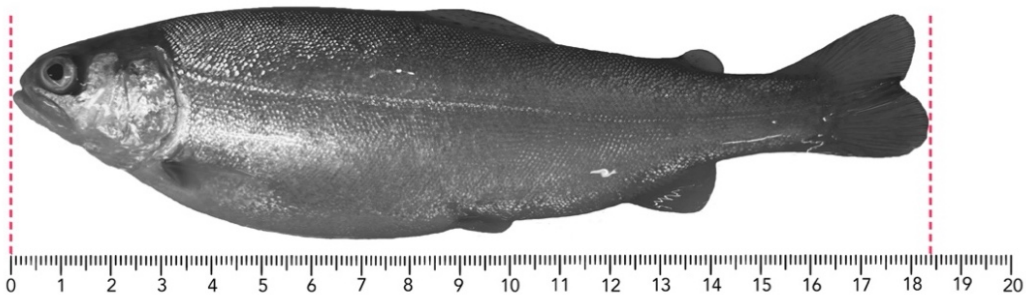

Figure 2. Example of how total length (cm) was measured (created by the author).

Fish were fed twice daily, where an automatic feeder would distribute the feed at 10 am and 14 pm for a duration of 30 minutes each time. The initial feed ration was at 1% of tank biomass per day, which was gradually increased to ensure the fish were fed to satiation. The feeding ration (in grams) was recorded daily throughout the trial. Feed waste was collected half an hour after feeding had ceased and faeces was collected two and a half hours after end of feeding. Feed waste was collected using flushing and faeces was collected using a combination of flushing and netting directly in the tank. When flushing, a valve at the bottom of the tank is briefly opened to allow water to empty out from the tank rapidly. By keeping a net beneath the valve, faeces in the water was captured. See figure 3 for illustration of tank set-up. During the trial, feed waste and faeces were stored between collections in a freezer at -20 C. After trial termination, feed waste from each tank was weighed and then dried at 103 ºC for 24 h in order to determine the dry matter content.

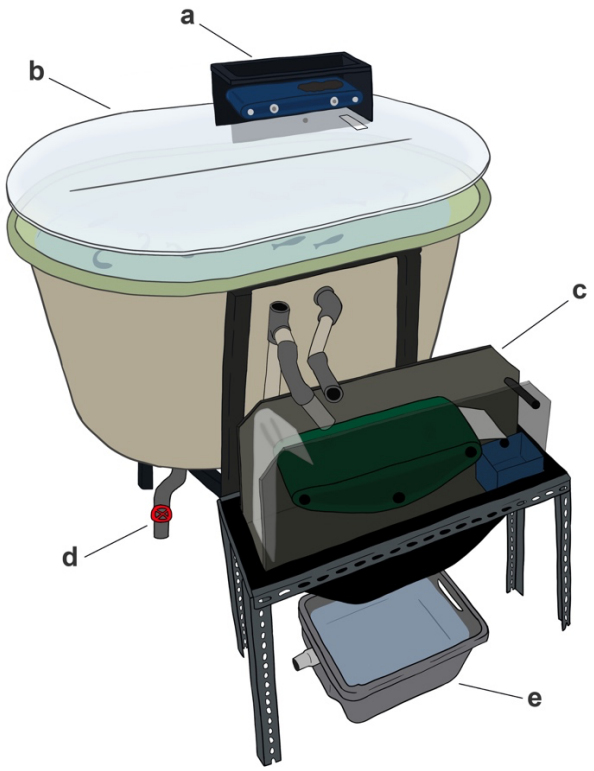

Figure 3. Illustration showcasing a) the automatic feeder, b) the covered trial tank, c) the collector, d) the flusher and e) the collection bucket (created by the author).

Daily measurements in four random experimental tanks were taken of dissolved oxygen (DO) levels (mg L^{-1}), temperatures (ºC) and water flows (L min^{-1}). DO levels and temperatures were measured using a portable probe (Hach HQ40D multimeter kit). Water flow was measured by timing the time it took for a 1 L glass beaker to fill up with water leaving the system, then dividing 60 seconds by this value. Throughout the trial period, DO averaged at 8.20 ± 0.51 mg L^{-1} and temperatures at 13.0 ± 0.1 ºC. Water flows were kept at 1.61 ± 0.23 L/min.

2.4 Blood sampling and preparation

Within 24 hours before the end of the trial, feeding was stopped. At the end of trial, fish from one trial tank at a time were netted and moved to be anesthetised in a 30 L bucket of water with 50 mg L^{-1} of Syncain (Syndel, Ferndale, Washington, USA) buffered with sodium bicarbonate. Fish were weighed and length was recorded.

Four randomly selected fish per tank (16 per diet) were euthanised in a 10 L bucket containing 200 mg L^{-1} of Syncain (Syndel, Ferndale, Washington, USA) buffered with sodium bicarbonate (Syndel, Ferndale, Washington, USA). Euthanised fish were included in the haematology and histology sampling. The remaining 21 fish from each tank were immediately after measurement, placed in a 40 L recovery bucket and, following recovery, released into a sectioned-off area of the holding tank (2500 L). Fish euthanised for the haematology and histology sampling had their caudal fins removed with surgical scissors, and a small amount of blood was drawn with a pipette.

For the haematocrit test, blood was collected in a heparinized microhematocrit tube. The tube was shut against a tray of sealing clay. Samples were thereafter centrifuged at 10.500 rpm for five minutes in a Hermle Z230H (BHG Hermle GmbH and Co., Gosheim, Germany). Haematocrit (HCT) levels were immediately read using a ruler, where the entire sample and the red blood cell layer lengths were measured. These values were then divided against each other to determine the relative percentage of red blood cell fraction.

$$Haematocrit\ level\ (\%) = \frac{Length\ of\ part\ with\ red\ blood\ cells}{Length\ of\ the\ entire\ sample} * 100 \qquad (1)$$

For histological samples and organ indices, these fish were dissected ventrally and viscera was removed and weighed. Then, the liver was weighed separately. Both measurements were recorded for later calculations of the Viscerosomatic index (VSI) and the Hepatosomatic index (HIS).

2.5 Physical pellet quality analysis

Bulk pellet density was recorded during the production of the feed pellets by measuring the weight of exactly 1 L of pellets. Five measurements per diet were taken, and expressed as g L^{-1}. For the pellet expansion rate, the diameter of ten pellets per diet was measured using an electronic calliper (Jula AB, Skara, Sweden). Calculation 2, where PD is the pellet diameter and DD the die diameter, was used.

$$Expansion\ (\%) = \left((PD - DD) * DD^{-1}\right) * 100 \qquad (2)$$

A hardness tester (Herkules M, Amandus Kahl GmbH & Co. KG, 21465 Reinbek, Germany), with a scale from 0-25 kgf, was used to determine the pellet hardness of experimental diets. 20 pellets per diet were tested in accordance with the manufacturer's protocol, and results were expressed in kgf.

The method used for the water stability test was adapted from Baeverfjord et al. (2006). In preparation, the pellets were sieved through a mesh of 3×3 mm to filter out any pieces that were small enough to fall through. Then, three replicates of 10 g each for each feed were placed in a previously weighed mesh basket, also with a mesh size of 3×3 mm and a diameter of approximately 10 cm. The basket with the pellets was placed inside a 600 ml beaker, to which 300 ml of tap water was previously added. After this, the beakers were secured in a water bath (Haake SWB 20, Haake Industries, Berlin, Germany), where temperatures were recorded before and at the end of each completed test. Here, the samples were shaken at 100 shakes per minute for 30, 90 and 180 minutes, respectively. After each time limit, mesh baskets were removed one at a time, gently dried with a paper towel, and the wet weight was documented. After this, samples were placed in an oven of 103 °C for 18 hours, after which the dry weight was also noted, and a residual dietary dry matter weight was calculated.

2.6 Calculations

Fulton's condition factor (K) was calculated using final body weights (FW) and total final lengths (FL), as average weights and lengths in each individual trial tank. The visceral weight (W_{VIS}) and the liver weight (W_{LIV}) were ascertained after the organ sampling. Averages from each tank were calculated and then used in the calculations for the hepatosomatic index (HIS) and the viscerosomatic index (VSI), respectively. The calculations were as follows:

$$K\ (g/cm^3) = \frac{FW}{FL^3} * 100 \quad (3)$$

$$HSI\ (\%) = \frac{W_{LIV}}{FW} * 100 \quad (4)$$

$$VSI\ (\%) = \frac{W_{VIS}}{FW} * 100 \quad (5)$$

The growth performance parameters were weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR). In calculations, final weight (FW) was again included, as well as start weight (SW), the duration of the trial in number of days (T) and feed intake (FI). These parameters were calculated according to Calculations 6-9.

$$WG\ (\%) = \left(\frac{FW-SW}{SW}\right) * 100 \quad (6)$$

$$SGR\ (\%\ day^{-1}) = \left(\frac{\ln FW - \ln SW}{T}\right) * 100 \quad (7)$$

$$FI\ ('as\ is'g) = \frac{dry\ feed\ given\ (g) - dry\ feed\ waste\ (g)}{DM\ of\ feed\ given\ (\%)} \quad (8)$$

$$FCR = \frac{FI}{FW-SW} \quad (9)$$

Apparent digestibility coefficients (ADCs) for dry matter and crude protein were calculated according to Cho et al. (1982):

$$ADC_{diet}\ (\%) = 1 - \left(\frac{F}{D} \times \frac{D_i}{F_i}\right) \times 100 \quad (10)$$

In Calculation 10, F is the nutrient percentage in faeces, and D is the nutrient percentage in the feed. D_i is the percentage of the marker in the analyzed diet, while F_i is the percentage of the marker in analyzed faeces samples.

2.7 Data management and statistical analysis

Data collected during the trial and at terminal sampling was stored in Microsoft Excel for Mac, version 16.89.1 (Microsoft Corporation, Redmond, WA, USA). The program was also used to perform calculations. Statistical analysis was done on physical pellet quality, body indices, growth performance and blood parameters using GraphPad Prism version 10.4.0 for Mac (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com). Analysis always comprised a one-way ANOVA, followed by Tukey's multiple comparison tests. The significance level was set at $P \leq 0.05$.

2.8 Ethical statement

The feed trial was performed in the Aquatic Facility, Department of Applied Animal Science and Welfare, SLU, Uppsala, Sweden. Experimental fish included in the trial were handled in full compliance with laws and regulations on procedures and experiments on live animals in Sweden, in accordance with the ethical permit issued by the Swedish Board of Agriculture (Registration number: 5.8.18-23275/2022).

3. Results

3.1 Nutritional composition of protein ingredients

The nutritional composition of FM and the alfalfa white protein concentrate can be found in Table 4. The WP concentrate contains crude fibre and NDF, in contrast to FM which contains no starch. Moreover, the chemical score of essential amino acids (EAA) shows WP has a higher content of all amino acids except for Isoleucine, Lysine and Methionine (Figure 4), expressed as g/kg.

Table 4. The nutritional composition of fish meal and white protein concentrate used in experimental diets is expressed as g kg-1 DM if nothing else is stated.

Diet	Fish meal	Alfalfa white protein
DM (%)	91.9	99.4
Ash	206.8	33.1
Crude protein	752.2	827.2
Crude fat	108.9	10.4
Crude fibre	ND	14.1
NDF	ND	6.1
GE (MJ kg^{-1} DM)	21.9	22.6
Sum of EAA	383.5	429.4
Essential amino acids		
Arginine	40,6	63.0
Histidine	16,7	26.1
Isoleucine	39,6	39.1
Leucine	51,1	78.4
Lysine	61,7	54.9
Methionine	23,6	17.2
Phenylalanine	29,2	53.0
Threonine	27,1	45.2
Valine	35,5	52.6

ND = not detected

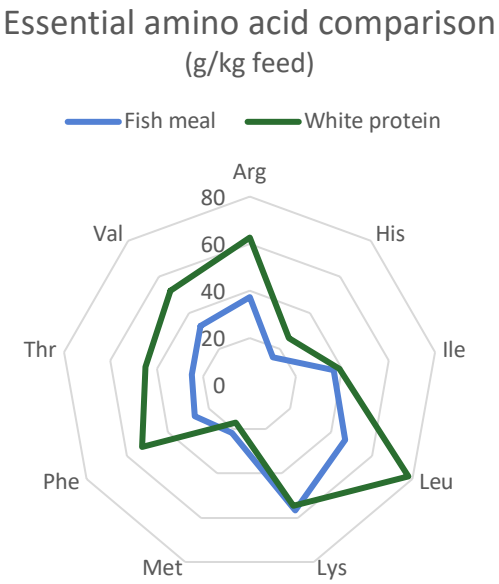

Figure 4. The chemical score of essential amino acids found in the white protein ingredient and fish meal used in the experimental diets.

3.2 Physical pellet quality analysis

The water stability tests indicate that including WP affected the water stability in all time categories tested (Appendix 3, Figure 12). The WP20 diet continuously had significantly higher water stability than all other feeds, except in the 180-minute test, where no significant difference was found between WP20 and CTRL. The WP5 and WP10 diets performed similarly to each other throughout each test and were consistently less water-stable than the CTRL diet (Figure 5).

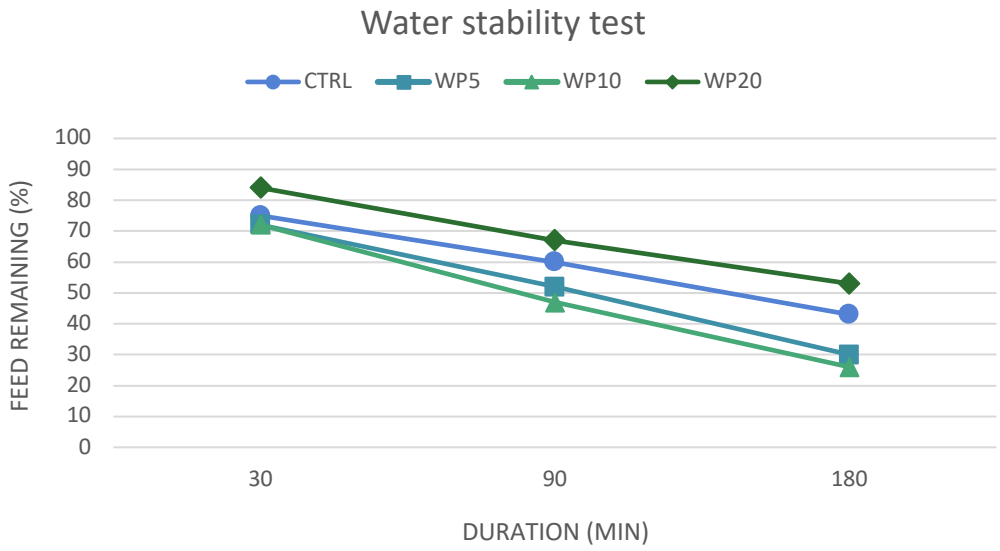

Figure 5. Water stability index (%) of experimental diets, after 30, 90 and 180 minutes.

All experimental diets showed similar bulk densities and expansion ratios (Appendix 2, Figure 11). However, the hardness test showed that the WP20 diet had significantly lower hardness than the CTRL diet. A summary of all pellet quality results can be found in Table 5.

Table 5. Summary of pellet quality analysis parameters tested.

Diet	CTRL	WP5	WP10	WP20	SEM	P_{ANOVA}
Bulk density (g L^{-1})	474.20	463.60	464.60	469.60	3.87	0.2637
Expansion ratio (%)	18.20	17.10	16.15	13.25	2.09	0.4006
Hardness test (kgf)	3.68^{a}	3.63^{ab}	3.20^{ab}	3.03^{b}	0.16	0.0173
Water stability test (%)						
30 minutes	74.67^{a}	71.67^{a}	71.67^{a}	84.33^{b}	1.25	0.0006
90 minutes	60.00^{a}	52.00^{b}	47.67^{b}	67.00^{c}	1.52	< 0.0001
180 minutes	43.67^{ac}	30.00^{ab}	26.33^{b}	52.67^{c}	2.64	0.0013

Different superscripts within each row indicate significant differences between diets ($P < 0.05$)
Bulk density, n=5; Expansion ratio, n=10; Hardness test, n=20; Water stability test 30, 90 and 180 minutes, n=3

3.3 Body indices and growth parameters

There were no significant differences in start weight between the treatments. Fish weight gain was generated by all four diets (see Figure 6), and no significant difference was found between fish fed CTRL, WP5 and WP10. There were also no significant differences in WG between fish fed WP5, WP10 and WP20. However, the numerically lowest WG was recorded for fish fed the WP20 diet and WG for the WP20 diet was significantly lower than for the CTRL diet ($P < 0.05$). WG varied more among tanks fed the CTRL diet.

Different inclusion levels of WP in diets did not result in varying HSI or VSI. However, fish fed the WP20 diet had a lower Fulton's K than the CTRL and WP5 diets ($P < 0.05$). Throughout the trial, fish mortality occurred in five of the tanks and was at 3.33% in each (one fish per tank). There were no significant differences in FI and SGR between the fish fed CTRL, WP5 and WP10 diets. Feed intake only varied between CTRL and WP20, where WP20 generated the lowest intake (Figure 7). Complete data for body indices and growth parameters can be found in appendices 4 (Figure 13), 5 (Table 8) and 6 (Table 9).

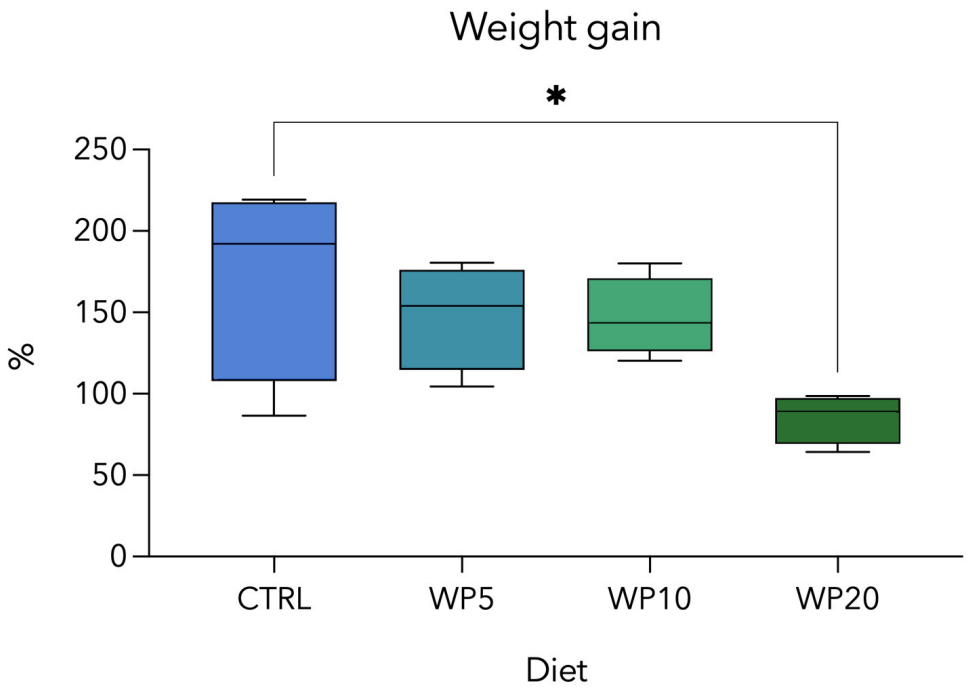

Figure 6. Average weight gains (WG) generated by the different experimental diets. Expressed in percentage, including SD and significant difference marked with asterisks (*).

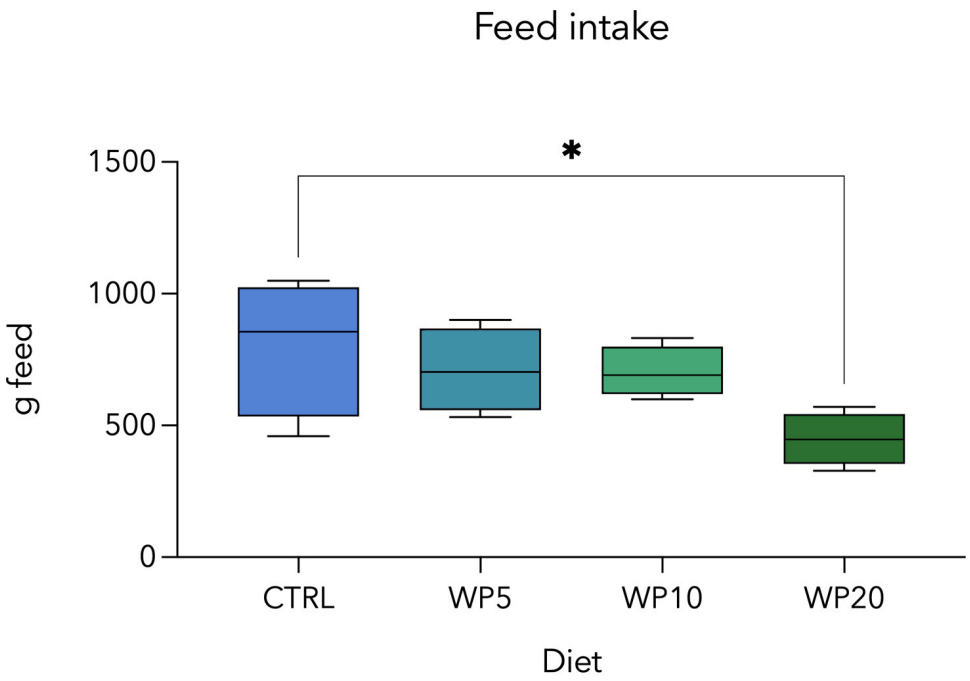

Figure 7. Feed intake at different inclusion levels of WP in experimental diets.

Similarly, the SGR of WP20 was lower than that of CTRL. However, the FCR did not show any differences between diets. A full summary of body indices and growth parameters can be found in Table 6. Moreover, tank-based summaries can be found in Appendices 7 and 8.

Table 6. Averages, standard errors of the means (SEMs) and P-values of analyzed body indices and growth performance parameters per experimental diet.

Diet	CTRL	WP 5	WP 10	WP 20	SEM	P_{ANOVA}
VSI (%)	16.06	15.25	16.60	21.87	1.54	0.0563
HSI (%)	2.02	1.73	1.81	2.21	0.17	0.3063
Condition factor (K)*	1.27^{a}	1.22^{a}	1.21^{ab}	1.13^{b}	0.02	0.0053
Weight gain (%)	172.56^{a}	148.27^{ab}	146.85^{ab}	85.29^{b}	16.68	0.0361
Feed intake (g feed)	804.8^{a}	709.7^{ab}	703.3^{ab}	447.9^{b}	77.02	0.0567
SGR (% day $^{-1}$)	2.09^{a}	1.92^{ab}	1.92^{ab}	1.31^{b}	0.15	0.0284
FCR (kg feed)	0.77	0.79	0.80	0.87	0.03	0.1385

Different superscripts within each row indicate significant differences between diets ($P < 0.05$)

* Fulton's condition factor (K)

3.4 Hematocrit levels

No significant differences in HCT levels were found between the CTRL, WP5, and WP10 diets (Appendix 7, Tables 10 and 11). However, a strong tendency toward significant differences was identified between CTRL and WP20 ($P = 0.0532$). See Figure 8 for a box-plot of the HCT results.

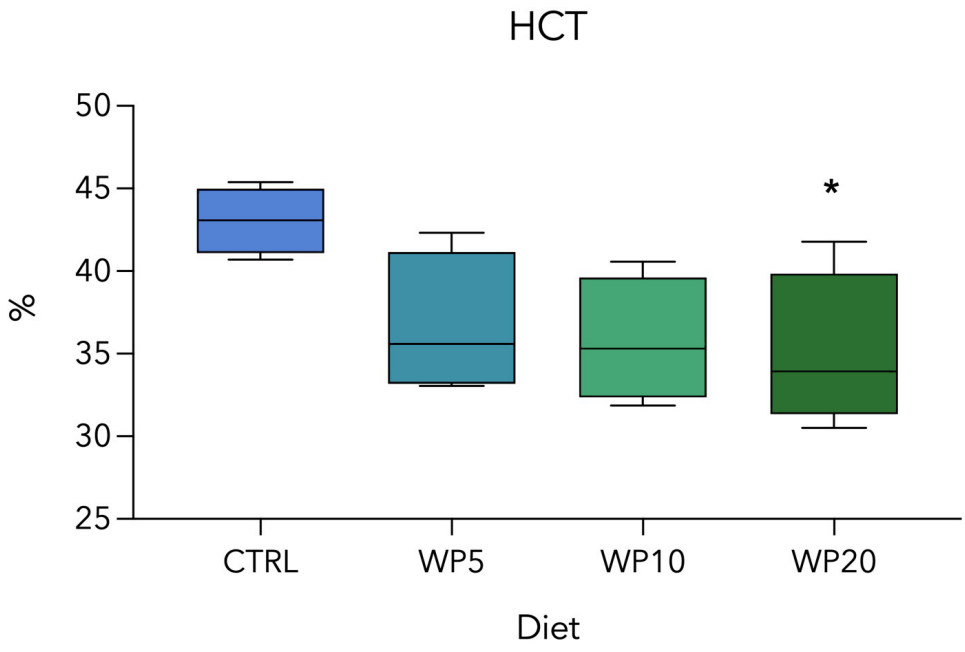

Figure 8. Box plot of hematocrit levels of different diets. Asterisks (*) indicate a strong tendency toward significant difference (P < 0.05).

3.5 Apparent digestibility

The ADC of dry matter showed no significant differences between the CTRL, WP5 and WP10 diets. However, the ADC of dry matter for WP20 was significantly lower than both CTRL and WP5. The CTRL and WP5 diets showed significantly higher ADC of crude protein compared to both WP10 and WP20. ADC results can be found in Figure 9 and Table 7.

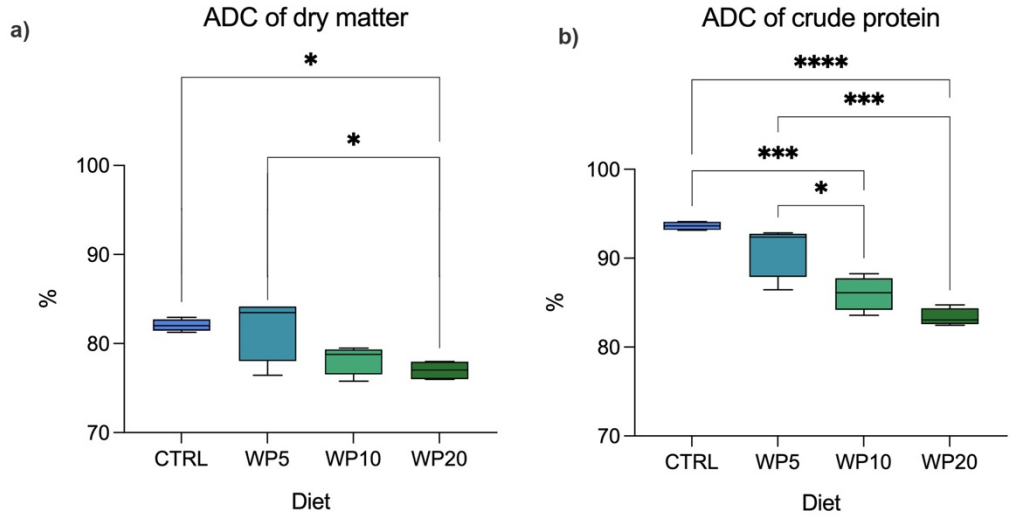

Figure 9. The ADC of a) dry matter and b) crude protein.

Table 7. Apparent digestibility coefficient results.

ADC (%)	CTRL	WP5	WP10	WP20	SEM	P_{ANOVA}
Dry matter	82.05^{a}	81.89^{a}	78.21^{ab}	77.00^{b}	0.89	0.0105
Crude protein	93.66^{a}	91.01^{a}	86.02^{b}	83.34^{c}	0.80	< 0.0001

Different superscripts within each row indicate significant differences between diets ($P < 0.05$)

4. Discussion

4.1 Physical feed quality

Feed pellets with higher water stability will remain intact longer, allowing fish more time to ingest them before they dissolve (Sørensen 2012). Increased water stability also means less nutrient leaching to the surrounding environment from any uneaten feed (White 2013). In the present trial, the WP20 diet had the highest water stability for all three time points, but the WP5 and WP10 diets sometimes exhibited lower water stability than the CTRL diet. This indicates that a higher WP inclusion is not necessarily correlated with increased water stability. Rather, the pre-extrusion of the WP20 diet is likely the leading cause for these differences. Based on this, pre-extrusion of feed pellets should be further explored to test its effect on physical pellet quality when including the alfalfa white protein concentrate in fish feed.

However, Chen et al. (2024) saw a significant increase in water stability when replacing FM in fish feed for rainbow trout with 10%, 15% and 20% of alfalfa nutritional concentrate compared to 0% and 5% inclusion. These results contradict those of the current trial, where the WP10 diet had the overall lowest water stability. Notably, Chen et al. (2024) performed the water stability test by soaking the pellets for 15 and 30 minutes, whereas the present trial used a modified method by Baevefjord et al. (2006), in a shaking water bath for 30, 90 and 180 minutes. Given this, we cannot exclude that WP inclusion below 20% has a negative effect on water stability. As there was no significant difference between CTRL, WP5 and WP10 in the 30-minute test, the longer time frames tested and the methodology used may be the reason for different results. Furthermore, as discussed by Glencross (2020), there may be many variations between the alfalfa product itself, which may also lead to different results.

No significant difference was found between the WP5 and WP10 diets compared to the CTRL diet in the remaining physical pellet quality tests. However, the pellet expansion ratio gradually decreased as higher levels of WP were included in the feeds. A sufficient expansion ratio is necessary to ensure pellet capacity for fat absorption and, thus, that the feed contains enough energy to meet nutritional

demands and sustain growth (Sørensen et al. 2010). Wheat starch is the main ingredient facilitating the expansion of extruded salmonid feed pellets, and other starches may have differing effects on expansion (Sørensen 2012). This was observed by Øverland et al. (2009), when pea protein concentrate was included in fish feeds which lead to decreased pellet expansions. This was likely due to pea starch being less gelatinization than wheat starch (Øverland et al. 2009). The WP diets in this trial contained lower levels of wheat starch than CTRL. Moreover, the WP concentrate acts as both a protein and starch component; thus, the expansion may be affected by the specific properties of the starch in WP. Temperature had to be slightly increased in the last barrel section for the WP20 diet and bar pressures successively increased with higher WP inclusion levels.

While none of the experimental diets in this trial had higher bulk density than the CTRL diet, the numerical increase in bulk density observed from 5% to 20% of WP content cannot be neglected. Usually, a more expanded pellet will have a lower bulk density, which directly affects the floating properties of feeds (Glencross et al. 2011). This correlation is found in the diets containing WP, where the WP5 diet has the highest expansion rate and the lowest bulk density, while the opposite is true for the WP20 diet. The CTRL diet strays from this pattern by numerically having the highest expansion rate and bulk density. A bulk density test was also performed by Chen et al. (2024), showing increased bulk density with higher levels of alfalfa nutritional concentrate.

The hardness test indicated that pellets become softer with increased WP inclusion levels. In a previous study by Aas et al. (2011), two experimental diets with varying physical pellet qualities were compared, showing that harder pellets likely led to prolonged gastric evacuation time, resulting in lower feed intake. Contradictory, however, Aas et al. (2020) saw no correlation between pellet hardness and feed intake when comparing three diets, again with varying physical pellet qualities. While there is a pattern of decreased feed intake for diets with reduced pellet hardness in this study, the feed intake may be impacted by other parameters than the hardness level.

Furthermore, water stability has been seen to be higher (Aas et al. 2011) and lower Aas et al. (2020) in pellets with increased pellet hardness. Both studies discuss that varying process conditions and ingredient inclusions may affect the physical pellet qualities (Aas et al. 2011, 2020). In this study, pellet hardness and water stability decreased progressively with increasing inclusion of WP in the CTRL, WP5 and WP10 diets. However, the WP20 diet completely diverges from the pattern, having simultaneously the lowest hardness level and the highest water stability. Previously, lower expansion ratios have been observed in diets with increased pellet hardness

(Sørensen 2012). This study shows an opposite pattern between hardness and expansion ratios. Thus, the results indicate that the combination of lower starch content, double extrusion, and low expansion ratio can lead to a feed that exhibits high water stability while being softer. The unusual combination of the observed results may be a unique trait of that specific feed formulation and processing method applied in this study.

4.2 Growth performance and apparent digestibility

No significant differences were found between the CTRL, WP5 and WP10 diets in any body indices and growth performance parameters tested. In contrast, this trial showed that fish fed with a diet containing WP at 20% had significantly reduced Fulton's K, weight gain, feed intake and SGR compared to the CTRL diet. The Fulton's K of the WP20 diet was also significantly below that of the WP5 diet. Notably, variation in weight gain and feed intake was greater for fish fed the CTRL diet, which may impact the results. Other studies have similarily to this study found that growth parameters are affected negatively by increased inclusion levels of alfalfa-derived ingredients (Ali et al. 2003; Coburn et al. 2021; Chen et al. 2024). Ali et al. (2003) saw that inclusion levels of alfalfa meals above 5% reduced growth performance and nutrient utilisation in Nile Tilapia. Yellow perch fed with alfalfa protein concentrate exhibited decreased SGRs compared to control and increased FCRs (Coburn et al. 2021). Chen et al. (2024) reported reduced growth at an alfalfa nutritional concentration of 10% and above. For SBM, further processing of the meal has been seen to lower ANF levels and increase protein content, resulting in a more viable protein source for fish feed (Alarape et al. 2024). Comparatively, the concentrated alfalfa WP extract used in this study has not greatly improved the parameters analysed by Ali et al. (2003), where an alfalfa meal was used.

Feed intake typically correlates with palatability and weight gain in fish, and both feed intake and weight gain are often the main indicators assessed to determine nutrient and energy utilisation in fish (Glencross 2020). As both weight gain and feed intake were lower in the WP20 diet, it is likely the fish have found it less palatable. However, based on our data, others factor as digestibility and possibly also antinutritional factors will affect the bioavailability, as discussed below.

In this trial, decreased digestibility of dry matter and crude protein was observed with increased levels of WP in the diet. Fish fed a diet containing WP at 5% and 10% showed similar ADC of dry matter as those fed the CTRL diet. The WP20 diet performed significantly below both the CTRL and WP5 diets. Similar results were found by Chen et al. (2024), and the authors discuss that indigestible carbohydrates may cause a reduced ability to digest dry matter. The alfalfa nutritional concentrate

used by Chen et al. (2024) had an NDF level of 11%, while the WP used in this trial had an NDF of 6.1%. Moreover, the WP10 and WP20 diets had substantially lower ADC of crude protein than the CTRL and WP5 diets. This indicates that, although these WP diets met protein requirements and the WP ingredient has a promising amino acid score, the fish digest the protein from the CTRL diet better than those containing WP. This may be caused by ANFs expected to be found in alfalfa, such as saponins, protease inhibitors, phytoestrogens and antivitamins, which could reduce the fish's ability to digest nutrients in feeds (Francis et al. 2001).

Chen et al. (2024) saw no significant difference in ADC of crude protein and argued that the poor growth seen in that study was unlikely caused by protease inhibitors. However, the effect of saponins on juvenile Japanese flounder (*Paralichthys olivaceus*) has previously been studied (Chen et al. 2011). The authors noted that increased levels of saponins seemed to negatively affect weight gain and ADC of crude protein, as well as feed intake in the initial four weeks of the trial. Although the quantification of ANFs has not been performed in this study, further feed trials with WP may consider doing so in the future.

These results indicate that a WP inclusion level of 10% may already be high enough to inhibit the digestive capability of rainbow trout and negatively affect the growth performance. However, other fish species, particularly omnivorous fish, may be better adapted to processing protein from alfalfa (Yanar et al. 2008; Chen et al. 2024). Yet, higher inclusion levels of WP could still work for rainbow trout if harmful traces of ANFs in alfalfa are reduced or removed. This assumes the feed is also considerably palatable for the fish, as this directly impacts feed intake (Glencross 2020).

Physical pellet quality properties brought on by differences in production parameters or the WP content may also impact the palatability and digestibility of the feeds. Aas et al. (2011) saw that rainbow trout fed a diet with higher water stability had better digestion of nutrients than those fed a feed with lower water stability. This is not observed in this trial. Furthermore, pre-extrusion of the microalgae *Nannochloropsis* in fish feed for Atlantic salmon (*Salmo salar*) has been seen to have no improved effect on nutrient utilisation (Liu et al. 2022). Pellet water stability was seen to likely improve with pre-extrusion of the WP ingredient, but more research should be done to explore the effects on nutritional utilisation.

4.3 Fish health and welfare

Knowledge of hematocrit levels, haemoglobin content, and leucocyte count is useful in detecting secondary stress responses in fish (Seibel et al. 2021). No significant difference was found in hematocrit levels in this study, but fish consuming diets containing WP all had lower hematocrit levels than CTRL. The values were within previously defined reference ranges (30.4-50.2% for females and 34.0-54.6% for males) (Řehulka et al. 2004). Still, all diets containing WP were in the lower part of these ranges, with HCT levels between 35.04-36.64%. These results are inconsistent with Chen et al. (2024), where HCT levels remained fairly level across all experimental diets, irrespective of alfalfa nutritional concentration content variations (averaging 45-46%). The CTRL diet in this study had an HCT level of 43.06%, which is similar to those observed by Chen et al. (2024).

Decreased HCT levels in fish can be associated with malnutrition or infections and cause anaemia (Seibel et al. 2021). It is important to consider, though, that lower functional HCT levels than those discussed so far have previously been observed (Pearson & Stevens 1990; Gallaugher et al. 1995). In a study on the effects of splenic erythrocyte reservoir release by Pearson & Stevens (1990), hematocrit levels normally occurred at around 20%. Moreover, MS-222 was not seen to affect the HCT levels much, but different types of handling caused significant increases to 37.9 ± 2.1% and 38.8 ± 0.5%, respectively (Pearson & Stevens 1990). This increase was attributed to the spleen releasing erythrocytes (a type of red blood cells) as a response to the stress stimuli, which occurred irrespective of MS-222 sedation (Pearson & Stevens 1990). It seems that all diets in this study measured high HCT levels considering the values observed by Pearson & Stevens (1990). If stress caused by handling during sampling is the underlying factor to increased HCT levels in this study, it is curious that fish fed diets containing WP showed lower HCT increase. As reduced growth and nutrient utilisation have been observed in these groups, it is possible that the lower stress response, measured as HCT levels, may be impacted by their health status or other unknown factors.

Further efforts are also underway to analyse the effect on gut histology as part of the current study. The ANFs of the WP have not been quantified in this study but have potentially impacted the analysed growth parameters. A previous study by Chen et al. (2011) saw that increasing levels of soybean saponins in the diet of juvenile Japanese flounder generated more damage to the distal intestine and back. The authors discuss that this might be due to the defoliating effect of saponins on intestinal mucosa Chen et al. (2011).

4.4 Future research

In this study, pre-extrusion seemed to impact the physical pellet quality, e.g. by improving water stability. The effect of different processing methods, such as pre-extrusion, on the physical pellet quality and nutrient utilisation, thus growth performance, is presently under-studied and should be researched further.

The environmental impact of including WP in diets should be assessed. The search for alternative protein sources in fish feed is largely driven by environmental sustainability concerns, making it an important factor to consider. Life cycle assessments (LCAs) are commonly used to evaluate seafood products and make the impact of different products comparable (Ziegler et al. 2016). There are also other environmental assessment methods, which may be useful as completion to an LCA (Couture et al. 2019).

Comparing feed intake between the four experimental diets can indicate feed palatability. However, the feed rations during the feed trial were adjusted based on the amount of feed waste. Real-time observational data may be beneficial in increasing the understanding of fish activity during feeding with diets containing varying WP levels. Behavioural studies can also be useful in determining the attractability of fish feeds containing WP.

In the future, it is likely that mixing of several protein sources to get a suitable and sustainable combination in fish feed will become more common. Therefore, levels of alfalfa WP in feeds may be a good complement in combination with other protein ingredients.

5. Conclusions

Alfalfa white protein extract could be considered for inclusion in feed at concentrations of 5% and 10% without significant changes to most growth and body indices parameters for rainbow trouts. However, this should be confirmed by longer exposure times and at different life stages. At a 10% inclusion level, there is already a significant decline in the digestibility of crude proteins, and an inclusion level of 20% WP further generated significant reductions in body indices and growth performance parameters. This may be due to the presence of antinutritional factors, higher content of indigestible carbohydrates in the alfalfa plant or lower palatability. However, the WP20 diet was the most water-stable, likely related to the pre-extrusion of this feed. The effect of pre-extrusion should be studied further. A reduction in expansion ratios and pellet hardness was seen with increased inclusion of WP, but not to a significant level. Fish had reduced HCT increases if fed experimental diets containing WP. This may indicate that WP harms fish health, likely due to ANFs, but further studies on fish health parameters, e.g. fish gut health, should explore this aspect.

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Popular science summary

Novel protein extract from green valleys meets aquafeeds

Aquaculture plays an increasingly vital role in supplying nutritious food to meet the needs of a growing global population. For some time, however, the industry has worked to improve the sustainability of fish feed production in fed finfish aquaculture. This is primarily due to the dependency on certain protein sources in feeds, such as fishmeal and soybean concentrates, which are associated with negative environmental outcomes. Hence, there is a desire to find new alternative protein sources.

White protein extract, derived through green biorefinery from the alfalfa plant, has been considered a promising protein source. A feed trial was conducted on the performance of the protein extract in fish feed for rainbow trout. At this stage, the growth and health of the fish were under study, as well as the physical pellet quality of the experimental diets. Four different diets were formulated, one reference similar to commercial feeds and three replacing fishmeal with increasing levels of the protein extract (5%, 10% and 20%).

The fish were seen to have similar growth performance regardless of being fed the reference diet or those containing 5% and 10% of the protein extract. However, at 20%, most growth parameters were negatively affected. Hematocrit levels in all groups were higher than normal levels observed in rainbow trout by previous studies, but no significant differences were found. Notably though, fish fed diets containing WP all had lower HCT level response. Analysis of pellet quality parameters showed that the diet containing WP at 20% had improved water stability than the other diets, likely due to this diet being pre-extruded. Pellet hardness and expansion ratios were seen to decrease with increased inclusion of WP.

Based on these results, it is possible that WP at 5% and 10% could be considered in fish feed for rainbow trout. Furthermore, the positive effect of varying extrusion parameters on some pellet quality parameters in feeds containing WP should be further studied.

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Appendix 1: Images of experimental diets

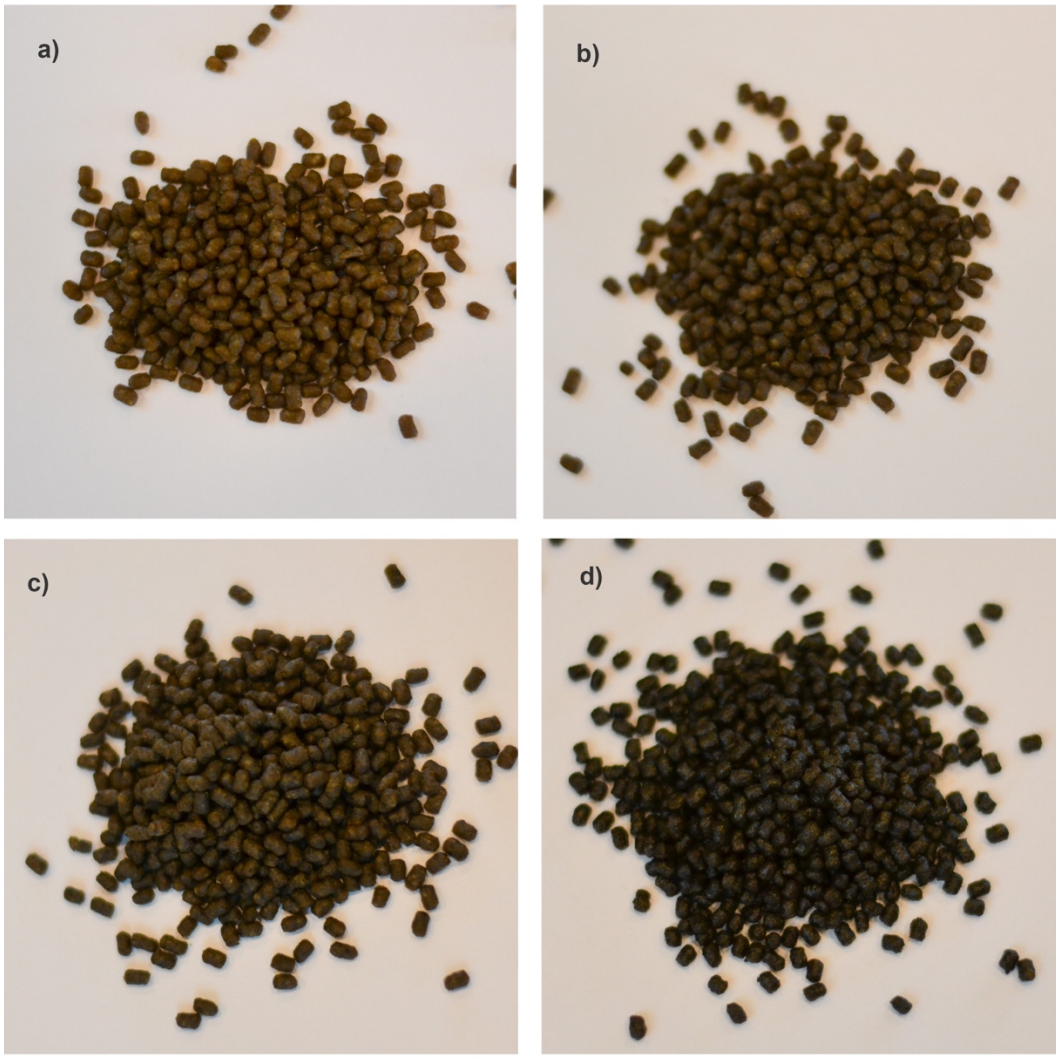

Figure 10. Close-up images of experimental diets, where inclusion levels of WP range from a) 0%, b) 5%, c) 10% and d) 20%. Photographed with a Nikon D3100 camera, using an AF-S Nikkor 50 mm lens.

Appendix 2: Bulk density, expansion ratio and hardness test

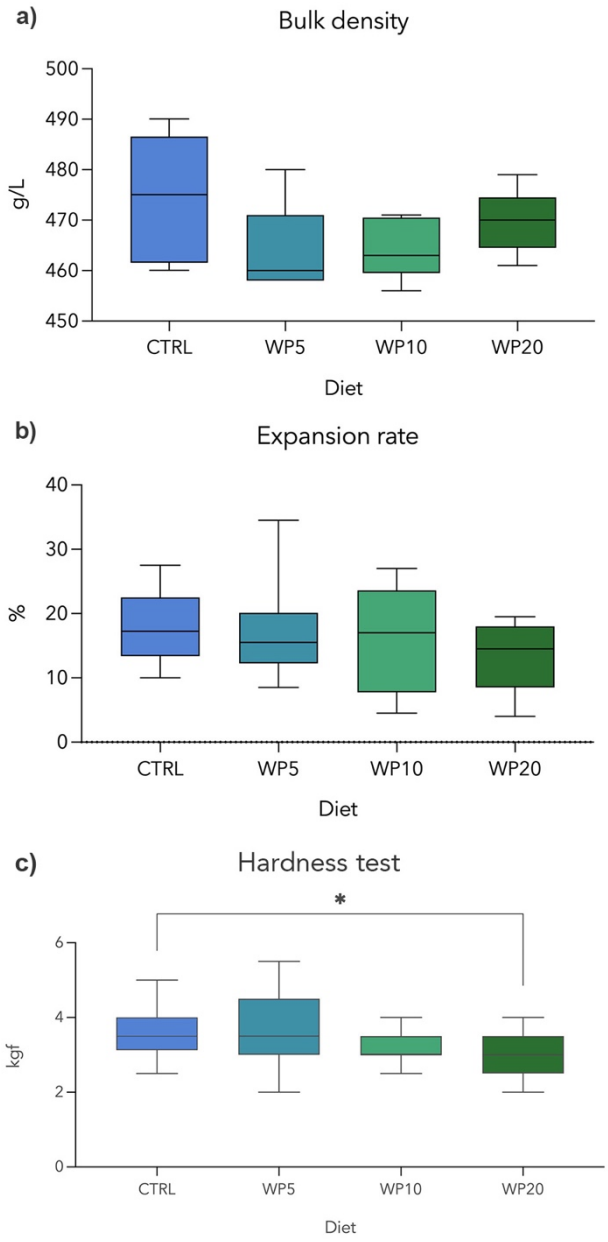

Figure 11. Box plots of a) bulk densities, b) expansion rates and c) pellet hardness of experimental diets.

Appendix 3: Water stability test

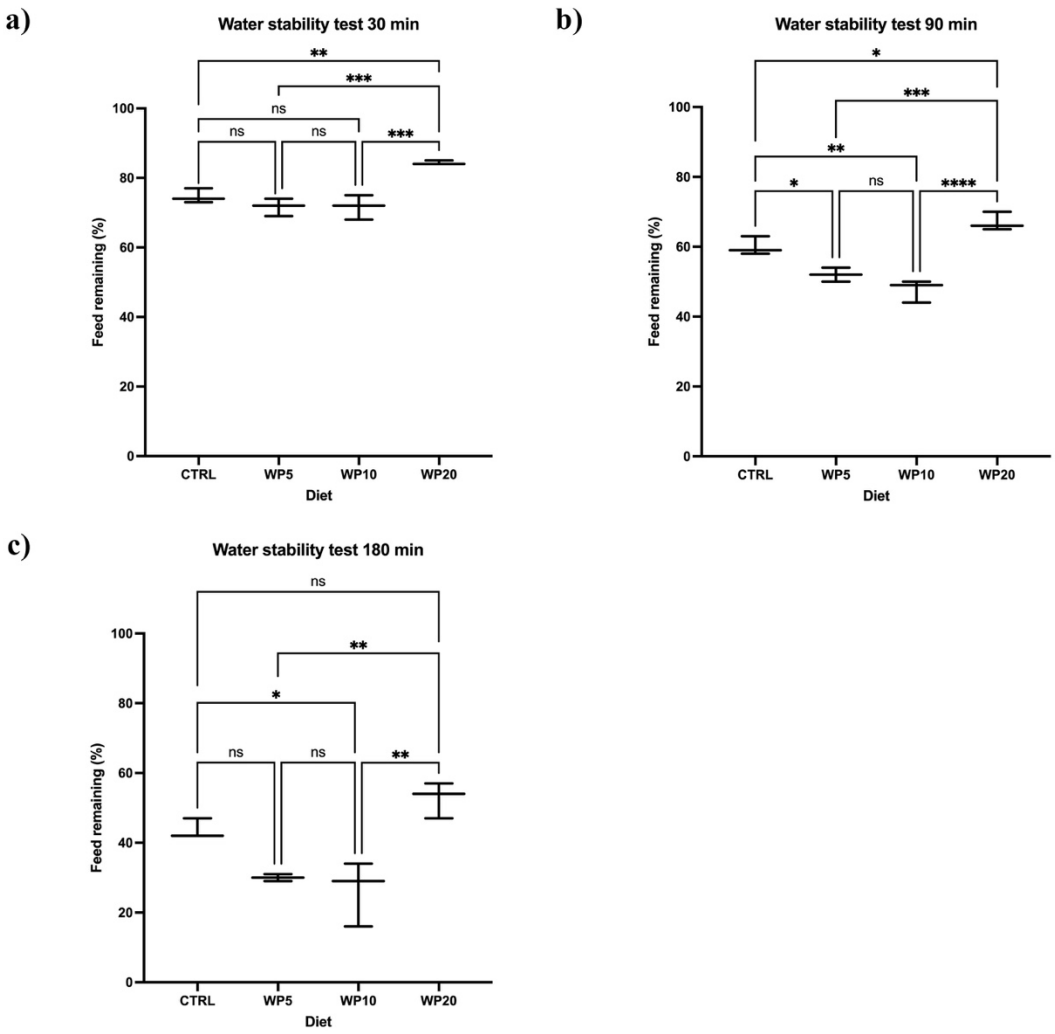

Figure 12. Water stability results for three different durations: a) 30 minutes b) 90 minutes and c) 180 minutes. Significant differences are marked by asterisks (*) and no significance by 'ns'.

Appendix 4: Body indices box plots

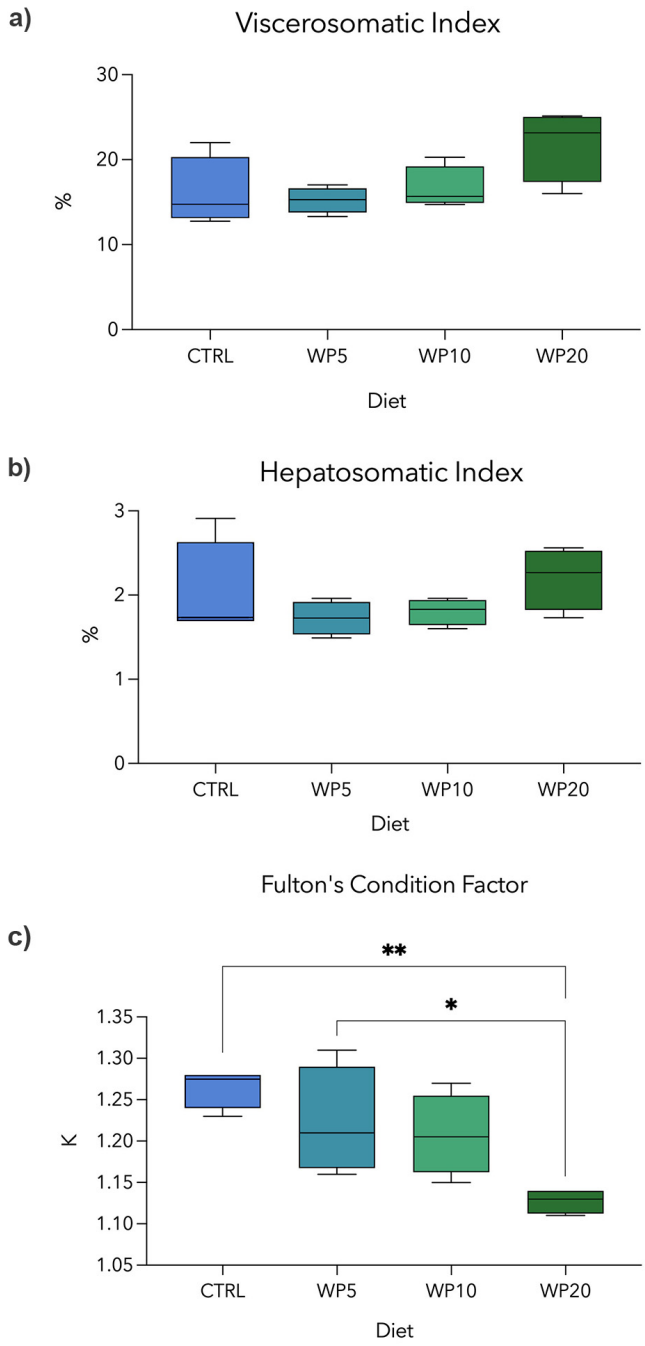

Figure 13. Box plots of a) VSI, b) HIS and c) Fulton's K, where significant differences between diets is marked with asterisks (*).

Appendix 5: Body indices data

Table 8. Mean measurements of four sample fishes per tank on trial termination day, including final weights (g) and lengths (cm), liver and visceral weights, and calculated VSI, HSI and K.

Tank	Diet	FW (g)	FL (cm)	W_{LIV} (g)	W_{VIS} (g)	VSI (%)	HSI (%)	K
1	WP20	48.3	16.4	0.80	8.23	17.04	1.66	1.10
2	WP5	54.2	16.7	0.80	7.30	13.47	1.48	1.17
3	WP10	59.2	17.1	0.90	8.68	14.67	1.52	1.19
4	WP5	60.0	16.9	0.98	8.40	14.01	1.63	1.25
5	CTRL	69.1	17.5	1,15	9.80	14.19	1.66	1.29
6	WP10	50.3	16.0	0.90	7.33	14.57	1.79	1.23
7	WP20	48.0	15.8	0.80	7.85	16.36	1.67	1.22
8	WP10	68.6	17.8	0.93	9.93	14.47	1.35	1.21
9	CTRL	59.0	16.7	0.93	7,80	13.22	1.57	1.28
10	WP10	54.5	16.4	0.90	7.50	13.76	1.65	1.25
11	CTRL	59.9	17.1	1.05	7,95	13.27	1.75	1.21
12	WP5	49.6	15.8	0.80	6.95	14.01	1.61	1.25
13	WP5	60.1	17.3	0.90	8.03	13.36	1.50	1.16
14	WP20	56.1	16.9	0.93	9.45	16.84	1.65	1.17
15	WP20	41.5	15.5	0.73	6.73	16.20	1.75	1.11
16	CTRL	79.8	19.1	1.15	8.70	10.91	1.44	1.15

Appendix 6: Growth parameters data

Table 9. Summary of average growth data obtained for each tank.

Tank	Diet	SW (g)	FW (g)	WG (g)	WG (%)	SGR (% day^{-1})	FCR	Mortality (%)
1	WP20	620.6	1144.5	523.9	84.4	1.30	0.83	0
2	WP5	588.6	1394.6	825.6	145.1	1.91	0.77	3.33
3	WP10	602.0	1685.4	1083.4	180.0	2.19	0.77	0
4	WP5	620.5	1631.0	1010,5	162.9	2.06	0.76	0
5	CTRL	639.0	1998.4	1359.4	212.7	2.43	0.70	0
6	WP10	625.4	1332.3	727.7	120.4	1.68	0.82	3.33
7	WP20	570.2	905.6	354.4	64.3	1.06	0.92	3.33
8	WP10	603.6	1418.8	835.3	143.2	1.89	0.84	3.33
9	CTRL	604.0	1641.8	1037.8	171.8	2.13	0.73	0
10	WP10	605.2	1476.1	870.9	143.9	1.90	0.78	0
11	CTRL	601.1	1083.6	502.5	86.5	1.33	0.91	3.33
12	WP5	598.2	1223.6	625.4	104.5	1.52	0.85	0
13	WP5	644.0	1806.9	1162.9	180.6	2.20	0.77	0
14	WP20	590.4	1144.3	553.9	93.8	1.41	0.83	0
15	WP20	634.7	1260.6	625.9	98.6	1.46	0.84	0
16	CTRL	640.1	2043.4	1403.3	219.2	2.47	0.84	0

Appendix 7: Hematocrit levels

Table 10. Average hematocrit based on four fishes per tank.

Tank	Diet	HCT (%)
1	WP20	30,52
2	WP5	37,66
3	WP10	31,86
4	WP5	33,55
5	CTRL	43,83
6	WP10	33,84
7	WP20	41,78
8	WP10	40,58
9	CTRL	42,33
10	WP10	36,74
11	CTRL	45,38
12	WP5	33,04
13	WP5	42,32
14	WP20	34,01
15	WP20	33,85
16	CTRL	40,69

Table 11. Average hematocrit levels per experimental diet, SEMs and P-values.

	CTRL	WP5	WP10	WP20	SEM	P_{ANOVA}
HCT (%)	43,06	36,64	35,76	35,04	1,86	0.0452

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